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Dynamic Regulation of SR Ca^{2+} Stores by STIM1 and Sarcolipin During Muscle Differentiation

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Abstract

During muscle development, the sarco/endoplasmic reticulum (SR/ER) undergoes remodeling to establish a specialized internal Ca^{2+} store for muscle contraction. We hypothesized that store operated Ca^{2+} entry (SOCE) is required to fill Ca^{2+} stores and is therefore critical to creating a mature SR/ER. Stromal interaction molecule 1 (STIM1) functions as a sensor of internal Ca^{2+} store content and an activator of SOCE channels. Myocytes lacking STIM1 display reduced SR Ca^{2+} content and altered expression of key SR proteins. Sarcolipin (SLN), an inhibitor of the SR calcium pump, was markedly increased in the muscle of mutant STIM1 mice. SLN opposes the actions of STIM1 by limiting SOCE, reducing SR Ca^{2+} content and delaying muscle differentiation. During mouse muscle development SLN is highly expressed in embryonic muscle, while the expression of STIM1 is upregulated postnatally. These results suggest that SOCE regulates SR/ER specialization and that SLN and STIM1 act in opposing fashions to govern SOCE during myogenesis.

Keywords

Calcium; STIM1; Sarcolipin; Myogenesis

Introduction

Cytosolic calcium $[Ca^{+2}]_i$ is a universal signaling molecule utilized for many cell processes including muscle contraction, cell proliferation, and regulation of gene expression (Berridge, 1996; Olson and Williams, 2000). How cells decipher calcium signals for specific cellular processes is currently under intense investigation, particularly in skeletal muscle. We have suggested that store operated calcium entry (SOCE) influences calcium-dependent gene expression in skeletal muscle by providing a sustained Ca^{2+} signal to enhance activation of signaling proteins such as calcineurin and calmodulin kinase (CamK)(Rosenberg et al., 2004). Calcium entry occurs through SOCE channels in response to SR Ca^{2+} store depletion that may be activated by developmental signals or in response to specific patterns of neurostimulation. Stromal interaction molecule 1 (STIM1), the calcium sensor and activator of SOCE channels, plays a critical role in the regulation of calcium-dependent gene expression in skeletal muscle(Stiber et al., 2008). STIM1 is located in SR/ER membranes where luminal EF hands sense changes in Ca^{2+} store content and activate SOCE channels (Zhang et al., 2005). Store depletion results in STIM1 aggregation and oligomerization. STIM1 can then migrate to areas underneath the plasma membrane to activate Orai1 and refill internal stores (Liou et al., 2007; Park et al., 2009). In skeletal muscle, SOCE displays

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rapid kinetics which may be required to rapidly reload SR Ca^{2+} stores following repetitive motor nerve stimulation (Launikonis and Rios, 2007; Stiber et al., 2008). We have therefore raised the hypothesis that specific factors regulate SOCE in muscle and account for the differences in SOCE between muscle and non-excitable cells.

Muscle differentiation occurs in a series of well orchestrated morphogenetic events that includes the formation of the sarcoplasmic reticulum (SR), a large highly specialized internal Ca^{2+} store (Franzini-Armstrong and Jorgensen, 1994; Rosemblit et al., 1999). Excitation contraction (EC) coupling is required in mature muscle in order to coordinate muscle contraction through SR calcium release and muscle relaxation by calcium uptake into the SR by the SR Ca2+-ATPase pump (SERCA)(Numa et al., 1990). SERCA1 is a skeletal muscle specific Ca^{2+} pump and exhibits faster kinetics than SERCA2B found in non-excitable cells (Vangheluwe et al., 2006). SERCA1 activity, and therefore SR Ca^{2+} content, is subjected to regulation by the accessory proteins phospholamban (PLB) and SLN (Periasamy and Kalyanasundaram, 2007). SLN physically associates with SERCA1 and decreases the Ca^{2+} affinity for SERCA, thus prolonging SR refilling time (Asahi et al., 2003; MacLennan et al., 2003). Mice lacking SLN exhibit expanded SR stores and augmented contractility, while overexpressing SLN in muscle is associated with delayed filling of the SR and impaired contractility (Tupling et al., 2002; Asahi et al., 2004; Babu et al., 2007b). Here, we show that SLN and STIM1 play opposing roles in the regulation of SOCE during muscle development. In this way, we suggest that specific temporal signals regulated by SLN and STIM1 govern SOCE during myogenesis in order to establish an internal SR/ER Ca^{2+} store.

Results

STIM1 Expression During Muscle Development

We previously established a loss of function model for STIM1 using a gene-trap approach that results in expression of a STIM1-LacZ fusion protein under the control of the endogenous STIM1 promoter (Stiber et al., 2008). The localization of the STIM1-LacZ fusion protein in heterozygous mice (*STIM1*$^{+/gt}$) can thus be used to determine the spatial and temporal expression patterns of endogenous STIM1 protein. We previously reported detecting the STIM1-LacZ fusion protein in all muscle groups that were harvested from adult heterozygous mice, but wished to investigate the temporal pattern of STIM1 expression during muscle development. We did not observe any STIM1 expression by β-galactosidase staining in somites or the developing limb buds at E10.5 and E12.5 days (Figure 1A–C). However, STIM1-LacZ was detected in the myotubes of the E15.5 embryo and later in developing post-natal muscle (Figure 1D–E). The pattern for STIM1 expression from STIM1-LacZ reporter mice was validated by measuring endogenous mRNA expression for STIM1 using real time PCR (Figure 1F).

STIM1, SOCE and SR Ca^{2+} stores

To understand how STIM1 loss of function influences Ca^{2+} signaling in primary myotubes, Fura-2 loaded myotubes isolated from WT and *STIM1*$^{-/-}$ mice were subjected to store depletion in a zero Ca^{2+} solution followed by readdition of 2 mM Ba^{2+}, as a surrogate of Ca^{2+}, to determine SR Ca^{2+} store content and SOCE respectively. Consistent with our previous studies, *STIM1*$^{-/-}$ myotubes exhibited a severe impairment in SOCE as the slope of the transient following the Ba^{2+} readdition was markedly reduced compared to WT myotubes (Stiber et al., 2008). Surprisingly, *STIM1*$^{-/-}$ myotubes also exhibited a significant reduction in SR Ca^{2+} content as determined by integrating the area under the curve (AUC) for the Ca^{2+} transient following CPA treatment (Figure 2A) (Bergling et al., 1998). These studies stand in contrast to many studies using STIM1 gene suppression or overexpression to

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manipulate SOCE in non-excitable cells. Activating or suppressing SOCE in these cells did not alter ER Ca^{2+} store content indicating a compensatory mechanism for maintaining filling of ER Ca^{2+} stores (Liou et al., 2005; Roos et al., 2005). Ca^{2+} stores were reduced in the $STIM1^{+/-}$ myotubes, but less so than the null myotubes. This intermediate phenotype did not reach statistical significance (not shown).

To further understand the defect in SR Ca^{2+} store content, we examined the SR morphology and expression profiles of key SR proteins. First, immunostaining for calsequestrin (CSQ), a calcium-binding protein of the SR, revealed no morphologic differences between $STIM1^{-/-}$ and WT myotubes (Figure 2B). Next, we profiled the mRNA expression levels of several molecules associated with Ca^{2+} handling in skeletal muscle (Figure 2C). No changes in expression were detected for STIM2, ryanodine receptor 1 (RYR1), L-type Ca^{2+} channel (LTCC) or canonical transient receptor potential channels (TRPC1–7) (data for TRPC2, TRPC 4–7 not shown). Similarly, no differences were observed for the expression of SERCA2b, mitsugumin 29 (not shown) or either cardiac or skeletal muscle isoforms for calsequestrin (cCSQ, sCSQ). Expression of SERCA1 was down regulated two fold which is consistent with our previous results (Stiber et al., 2008). In contrast, the mRNA and protein levels of sarcolipin were significantly upregulated (5–7 fold) in $STIM1^{-/-}$ myotubes (Figure 2C). Based on these studies we directed our focus to SLN as a possible factor that may contribute to the reduced SR Ca^{2+} stores observed in $STIM1^{-/-}$ myotubes.

Temporal Expression of SLN and STIM1

In situ hybridization revealed that SLN mRNA expression can be found in somites and all muscle tissues during embryonic muscle development (Figure 3) but then is restricted predominately to the atria and soleus muscles in adult muscle (not shown). Interestingly, SLN and STIM1 exhibit distinct temporal patterns of expression in skeletal muscle; SLN is highly expressed in embryonic muscle but less so in neonatal and adult muscle (Figure 4). In contrast, STIM1 is abundantly expressed in neonatal and adult muscle (Stiber et al., 2008). These findings raise the possibility that SLN and STIM1 may play alternate roles in the regulation of SOCE during specific phases of muscle differentiation and that downregulation of SLN expression signals the induction of SOCE and SR maturation.

SLN and SOCE

To further understand the relationship between SLN and STIM1, we generated two stably transfected lines of C2C12 cells expressing SLN as well as control cell lines stably expressing YFP and empty vector. SLN myotubes (S1 and S2) displayed an increase in basal cytosolic Ca^{2+} consistent with previous studies (Odermatt et al., 1998; Asahi et al., 2003), while exhibiting marked reductions in SR Ca^{2+} store content and SOCE compared to control myotubes (C1 and C2) (Figure 5A). In contrast, SOCE was augmented in myotubes transiently transfected with a short hairpin RNA (shRNA) plasmid designed to silence endogenous SLN (Figure 5B). These results suggest that SLN limits SOCE in myogenic cells and was associated with decreased expression of STIM1 protein in these cell lines (Figure 5D). To determine if SLN-dependent inhibition of SOCE was the consequence of reduced expression of STIM1, we expressed STIM1 plasmid in the SLN-overexpressing myotubes. Here, STIM1 was able to fully rescue SOCE in SLN-overexpressing myotubes (Figure 5C). SLN over expression was associated with a decrease in STIM1 expression, but interestingly, no changes were observed in the expression of SERCA1 or calreticulin in SLN-overexpressing myotubes indicating that the expression of SR/ER proteins of these myotubes was largely unchanged from control cells (Figure 5D).

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SLN and Muscle Differentiation

SLN-expressing myotubes appear proliferate, fuse and assemble myotubes in a fashion similar to GFP-expressing myotubes (Figure 6A). SOCE provides Ca^{2+} entry needed to maintain NFAT/MEF2 dependent transcriptional activity during muscle differentiation (Stiber et al., 2008). We reasoned then that SLN-overexpressing myotubes which express a defect in STIM1-dependent SOCE should also have a defect in muscle differentiation. Here, we transfected SLN-overexpressing and control myotubes with reporter constructs for several muscle specific promoters: myoglobin, desmin and slow troponin I. To control for changes in basal transcriptional activity, we also transfected these cells with a TATA reporter construct. While there was no change in activation of a basal TATA promoter, promoter activity for desmin, slow troponin and myoglobin were markedly reduced in the myotubes overexpressing SLN (Figure 6B). These results suggest that SLN overexpression delayed the activation of promoters for several genes known to be associated with muscle differentiation. We validated these reporter studies by showing that myoglobin expression was significantly reduced by Western blotting in SLN-overexpressing myotubes compared with controls. A similar reduction in expression was seen for NFATc1, a known transcriptional target of calcineurin/NFAT signaling (Figure 6C)(Zhou et al., 2002). The effect of SLN overexpression on SERCA inhibition was evidenced by changes to calcium transients. SLN overexpression resulted in reduced amplitude and prolonged duration of the calcium transients in response to KCl (40 mM) depolarization indicating that refilling of the SR/ER by SERCA1 was diminished. Moreover, caffeine (20 mM) stimulation of SLN myotubes revealed a marked reduction in the amplitude of calcium transients compared to WT myotubes (Figure 6D). These data provide functional evidence supporting the notion that SLN overexpression suppressed Ca^{2+} signaling and reduced SR Ca^{2+} stores in myotubes. When considered along with the developmental expression pattern, these data suggest that SLN blocks the maturation of Ca^{2+} signaling in embryonic muscle.

Discussion

While the importance of store operated Ca^{2+} entry (SOCE) in non-excitable cells has been recognized for decades, its importance in skeletal muscle has only recently been clarified. In the present study, we extend our previous work to show that internal Ca^{2+} stores from *STIM1*$^{-/-}$ myotubes were markedly diminished compared to WT myotubes. These findings stand in contrast to other studies wherein altering SOCE in non-excitable cells had minimal impact on Ca^{2+} store content (Mercer et al., 2006; Soboloff et al., 2006; Jousset et al., 2007). To reconcile differences in our data from skeletal myocytes compared to non-excitable cells, we show that the molecular makeup of the SR/ER of *STIM1*$^{-/-}$ myotubes is different from WT myotubes as SERCA1 was significantly reduced and SLN was substantially upregulated. These findings allowed us to hypothesize that a delay in the maturation of the SR/ER of *STIM1*$^{-/-}$ myotubes results in a reduction of the size of SR Ca^{2+} store and altered myogenic signaling. In fact, we suggest that STIM1 and SLN coordinate SOCE and therefore SR formation during muscle differentiation. These studies introduce a novel role for STIM1-SOCE in muscle differentiation and reveal SLN as a novel regulator of SOCE in muscle.

SLN is a small single pass transmembrane protein located in the SR of skeletal muscle. Because SLN inhibits SERCA1 activity, we wondered whether SLN would influence STIM1-dependent SOCE. We show that SLN overexpression in myotubes reduced SOCE, reduced SR Ca^{2+} content and delayed muscle differentiation. To explain these findings, we show that STIM1 is reduced in SLN-overexpressing myotubes, and reintroduction of STIM1 into SLN myotubes can rescue the defect in SOCE. Taken together our findings are consistent with a model in which SOCE is tightly coordinated during different phases of muscle development (Figure 7). In support of our model, we show that STIM1 and SLN are

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expressed in skeletal muscle in specific temporal patterns: SLN is expressed in early embryonic muscle whereas STIM1 is expressed in later stages of fetal muscle (E15.5) and postnatal muscle. We therefore propose that SLN blocks the filling of SR stores during early phases of myogenesis which may serve to prevent Ca^{2+} overload and defer muscle differentiation until later stages. In contrast during postnatal muscle development, SLN is limited to fibers in the soleus muscle and STIM1-dependent SOCE emerges to refill SR stores and promote muscle differentiation.

To better understand the SLN/STIM1 relationship, we tested our model with cultured myotubes and found that SLN overexpression in myotubes limits Ca^{2+} entry following store depletion, whereas myotubes expressing a shRNA plasmid for SLN demonstrate greater SOCE than controls. These results indicate that the influence of SLN on Ca^{2+} signaling may involve not only inhibiting SERCA1 activity but also the size and function of the SR Ca^{2+} pool by altering STIM1 expression. These results are consistent with the known function of SLN in atrial and skeletal muscle (MacLennan, 2000; Minamisawa et al., 2003; Babu et al., 2007a). In fact, atria of $SLN^{-/-}$ mice exhibit greater SR Ca^{2+} stores and enhanced contractility (Babu et al., 2007b). In contrast, SLN transgenesis in the heart and skeletal muscle impairs the ability of SERCA1/2 to refill of internal stores resulting in a prolonged Ca^{2+} transient and impaired contractility (Asahi et al., 2003; Asahi et al., 2004). In our current studies, we show that SLN-myotubes exhibit markedly perturbed Ca^{2+} signaling. For example, KCl-depolarization of the SLN-myotubes resulted in Ca^{2+} transient with diminished amplitude but prolonged duration compared to WT myotubes. These results indicate SLN limited the SERCA1 from re-sequestering Ca^{2+} into the SR/ER stores efficiently. Moreover, we show that reduced STIM1 expression impairs SOCE activation in SLN-myotubes. We propose that the changes in Ca^{2+} signaling by SLN overexpression delayed muscle differentiation as indicated by muscle specific gene expression studies.

We have proposed that the fundamental role of SOCE in muscle is to coordinate Ca^{2+}/calmodulin-dependent gene expression programs governed by CamKII, calcineurin and possibly the calmodulin-binding transcription activator (CAMTA) family of transcription factors (Han et al., 2006; Song et al., 2006). In fact, STIM1-dependent SOCE is needed to replenish SR Ca^{2+} stores following repetitive neuromuscular activity(Launikonis and Rios, 2007). Signaling molecules such as CamKII and calcineurin would then interpret changes in frequency of Ca^{2+} oscillation to activate Ca^{2+} dependent gene expression. STIM1 deficient myotubes, which manifest a defect in repetitive Ca^{2+} signaling, exhibit impairment in calcineurin/NFAT signaling(Stiber et al., 2008). Notably, we found that SLN was upregulated in $STIM1^{-/-}$ muscle and may represent a marker for immature skeletal muscle. Interestingly, SLN-myotubes exhibit reduced STIM1 expression, reduced SOCE, and delayed muscle differentiation. When considered together, these results are consistent with the idea that SLN limits SOCE during early phases of differentiation in order to prevent premature differentiation of muscle. We further interpret these studies as evidence that $STIM1^{-/-}$ mice exhibit a delay in postnatal muscle development.

Augmented Ca2+ entry through SOCE has also been implicated as a cause for Ca^{2+} overload in several different forms of muscular dystrophies (Boittin et al., 2006; Edwards et al., 2010). Abnormal Ca^{2+} entry activates calpains, Ca^{2+}-dependent proteases, which contribute to degeneration of dystrophic muscle. This abnormal Ca^{2+} entry has been characterized as both store operated and stretch activated in dystrophic fibers (Franco and Lansman, 1990; Vandebrouck et al., 2002a). In fact, SOCE controlled by STIM1 was recently shown to be upregulated in *mdx* muscle fibers which lack dystrophin (Edwards et al., 2010). Along the same lines, several recent studies have implicated transient receptor potential channels (TRPC) as contributing to abnormal Ca^{2+} entry in dystrophic fibers (Vandebrouck et al., 2002b; Vandebrouck et al., 2007). Given that STIM1 is also known to

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regulate TRPC1 channels, it may be that TRP channels participate in establishing a SOCE complex in *mdx* muscle fibers (Yuan et al., 2007). We have favored the idea that upregulation of SOCE in different myopathies may represent a compensatory response to induce favorable changes in gene expression and accommodate the muscle damage (Stiber and Rosenberg, 2011). Squire et al. demonstrate that rescuing dystrophic phenotype with inducible utrophin expression is not accompanied by changes in the SOCE complex (Squire et al., 2002). Thus it remains to be seen if SOCE represents a pathologic mediator or mitigator of degeneration in dystrophic muscle.

In the present work, SLN is shown to block SOCE and impair muscle differentiation. These results raise interesting possibilities regarding the role of SLN in the regulation of SOCE during muscle regeneration and as a modifier of skeletal myopathies. Nemaline myopathies are a group of non-dystrophic myopathies resulting from a mutation in the thin filament protein nebulin. Mice lacking nebulin or patients with nemaline myopathy exhibit severely reduced muscle contractility which may be attributed to the accumulation of nebulin rods and alterations in Ca^{2+} signaling. Upregulation of SLN may represent one potential mechanism underlying the reduced contractility in the nebulin KO mice (Gokhin et al., 2009). In a similar fashion SLN is upregulated in an animal model of dysferlinopathy (Campanaro et al., 2002). In the present work, we show that SLN is upregulated in the muscle of *STIM1*$^{-/-}$ mice. It will be important to know whether STIM1 and SOCE are altered in nemaline myoapthies and dysferinopathy. We would speculate that STIM1 expression is down regulated resulting in diminished SOCE. In this way, SOCE dependent muscle differentiation would be limited and therefore contribute to muscle weakness associated with muscle regeneration. We suggest that SLN's influence on SR Ca^{2+} stores extends beyond SERCA1/2 inhibition but also involves limiting SOCE in immature myotubes. In fact, the changes in SLN/SERCA1 expression and the reduced SR Ca^{2+} stores observed in STIM1 mutant mice reflect a delay in muscle differentiation that involves the SR maturation process. During normal myogenesis or muscle regeneration, SLN and STIM1 may function interdependently to coordinate muscle differentiation in a regulated manner to prevent Ca^{2+} overload.

In conclusion, the present findings provide initial experimental evidence that SLN and STIM1 act to coordinate SR maturation and muscle differentiation. That SLN and STIM1 are dysregulated in several neuromuscular disorders raises the possibility that these control mechanisms govern SOCE during post-natal myogenesis. In this way, SOCE may be upregulated in order to promote muscle differentiation in regenerating muscle fibers. Based on these ideas, we suggest that modulation of STIM1-dependent SOCE may represent a future strategy for the treatment of various skeletal myopathies.

Experimental Procedures

β-galactosidase staining

Mouse tissues were dissected with cold PBS and immediately fixed with 2% paraformaldehyde and 0.2% glutaraldehyde. After rinsing the tissues with rinsing solution (5 mM EGTA, 0.01% deoxycholate, 0.02% NP40, 2 mM $MgCl_2$) 3 × 15 minutes at room temperature, the tissues were incubated in the dark with staining solution (5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, 5 mM EGTA, 0.01% deoxycholate, 0.02% NP40, 2 mM $MgCl_2$, 1 mg/mL X-gal solution) at room temperature until desired intensity was reached. The specimens were then washed with PBS, post-fixed with 4% PFA, and then stored in 70% ethanol. Paraffin sectioning of the stained tissues was performed by standard methods, and sections were subsequently stained with haematoxyllin.

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Cell Culture and Primary Myotube Isolation

C2C12 cells were grown in Dulbecco's modified media (DMEM) supplemented with 20% fetal bovine serum and 100 IU/ml penicillin and 100ug/ml streptomycin. Cell differentiation into myotubes took place over five days while cultured in media containing DMEM containing 2% horse serum, 100 IU/ml penicillin and 100 μg/ml streptomycin, 10 μg/ml transferrin, 10 μg/ml regular insulin and 50 mM HEPES buffer. In experiments involving stably transfected C2C12 cell lines, cells were selected using the G418 resistance (G418 concentration 800 μg/ml). Primary myocytes were isolated from neonatal mice by digestion in type I collagenase (Worthington) as previously described (Stiber et al., 2008).

Western blotting and Immunohistochemistry

Dissected muscles from euthanized mice were homogenized using a glass mortar and pestle in either 1% Triton lysis buffer containing 1% Triton X-100, 150 mM NaCl, 50 mM Tris pH 8, and protease inhibitor (Complete Mini, Roche) or lysis buffer containing 50 mM Tris pH 7.6, 500 mM NaCl, 0.1% sodium dodecyl sulfate (SDS), 0.5% deoxycholate 1% Triton X-100, and protease inhibitor. Protein concentrations were measured by the Bradford assay for equal loading and subsequent analysis by SDS-PAGE and Western blotting. For SDS-PAGE of sarcolipin, a 16% gel was used. Rabbit polyclonal antibodies to sarcolipin, calsequestrin, calreticulin, beta-actin and GAPDH were obtained from Abcam (Cambridge, MA). Mouse monoclonal antibody to STIM1 was obtained from BD Biosciences. Myoglobin antibody was obtained from Dako (Carpinteria, CA), and NFATc1 antibody was obtained from Cell Signaling Technology (Danvers, MA). Western blotting signals were detected by ECL Plus (GE-Amersham) and quantitated by densitometry. For immunofluorescence studies, myotubes were grown on glass bottom plates (MatTek, Ashland, MA) and the fixed in ice cold methanol for 10 min prior to addition of primary antibody.

Cell Transfection and Luciferase Assay

C2C12 myoblasts were plated at 10^5 cells per well of a 6 well dish at least 12 hours prior to transfection. Cells were transfected with 1.6 μg of NFAT reporter plasmid with Fugene (Promega). Cells were differentiated for four days in 2% horse serum and then asssayed for luciferase and ϐ-galactosidase from whole cell extracts as previously described (Wu et al., 2000).

In Situ Hybridization

For *in situ* analysis of embryonic expression, embryos were harvested at E9.5, E10.5 and E15.5, fixed in paraformaldhyde, and incubated with *in vitro* transcribed DIG labeled probes for SLN. Hybridization with sense and anti-sense probes was performed to identify specific mRNA transcripts.

Real Time PCR

Embryonic tissue was collected from timed matings, and developing muscle was isolated at E10.5, E15.5 and P2. Myotubes from SLN and control cell lines were differentiated for 5 days and harvested using the TRIzol (Invitrogen). The purity of the isolated total RNA was determined by gel electrophoresis and quantity was determined using a nanodrop spectrophotometer. cDNA was synthesized using a first strand synthesis kit (Roche) by standard techniques. Time resolved PCR was accomplished using the Taqman system with primers from ABI Biosystems for the indicated genes. Results reflect triplicate experiments. An unpaired Student's *t* test was performed comparing the experimental gene with 18S and GAPDH.

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Ca^{2+} Imaging

Myoblasts were plated and differentiated on glass bottomed culture plates coated with collagen. Cells were loaded with Fura-2 (Molecular Probes) for measurements of Ca^{2+} transients as previously described(Stiber et al., 2008). To determine the SR Ca^{2+} store content, the area under the curve for Ca^{2+} transients was determined for myotubes stimulated with the SERCA pump inhibitor cyclopiazoic acid (CPA 100 μM) in a zero Ca^{2+} bath. Results from at least 3 independent experiments were averaged for a minimum of 50 total cells.

Animal Care and Use

Experiments involving mice were performed according to NIH policies outlined in the Guide for Care and Use of Laboratory Animals. All protocols for animal research were reviewed by the Institutional Animal Care and Use Committee of Duke University Medical Center.

Statistical Analysis

Data are presented as means ± standard errors of the means. An unpaired Student's *t* test was used for comparison between two groups, and values of $p < 0.05$ were considered significant

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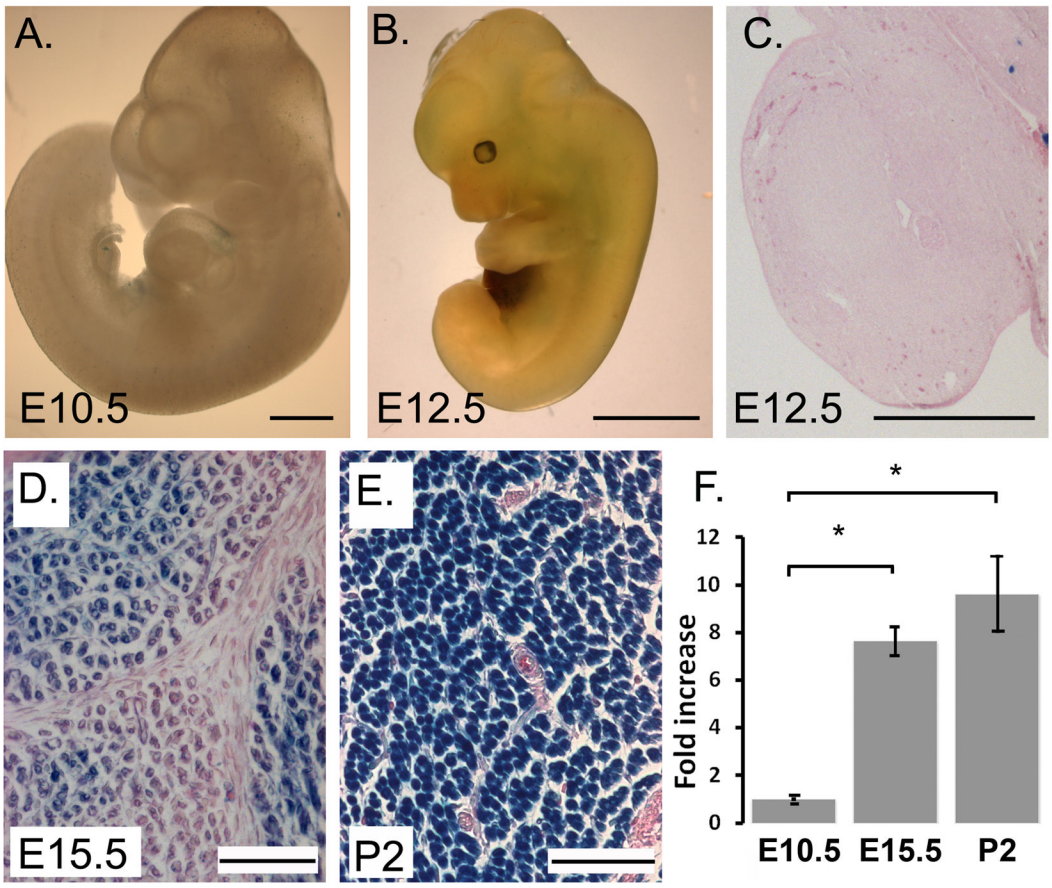

Figure 1.

STIM1 Expression During Muscle Development. A gene-trap approach was used to generate a STIM1-LacZ fusion protein under the control of the endogenous STIM1 promoter. The localization of the STM1-LacZ fusion protein in mice heterozygous for the gene-trap allele was used study the spatial expression pattern of STIM1. We did not observe any STIM1 expression by ϐ-galactosidase staining in somites or the developing limb buds (panels A–C). STIM1 expression was seen at E15.5 and later in developing myotubes and myofibers (panel DE). (Scale bars = 100 μM panels A–B, 50 μM panels C–E). mRNA was isolated from muscle at E10.5, E15.5 and P2, and STIM1 specific primers used for real time PCR. Quantification is provided for relative expression levels of STIM1 mRNA (n=4 mice at each time point, * $p < 0.05$) (panel F).

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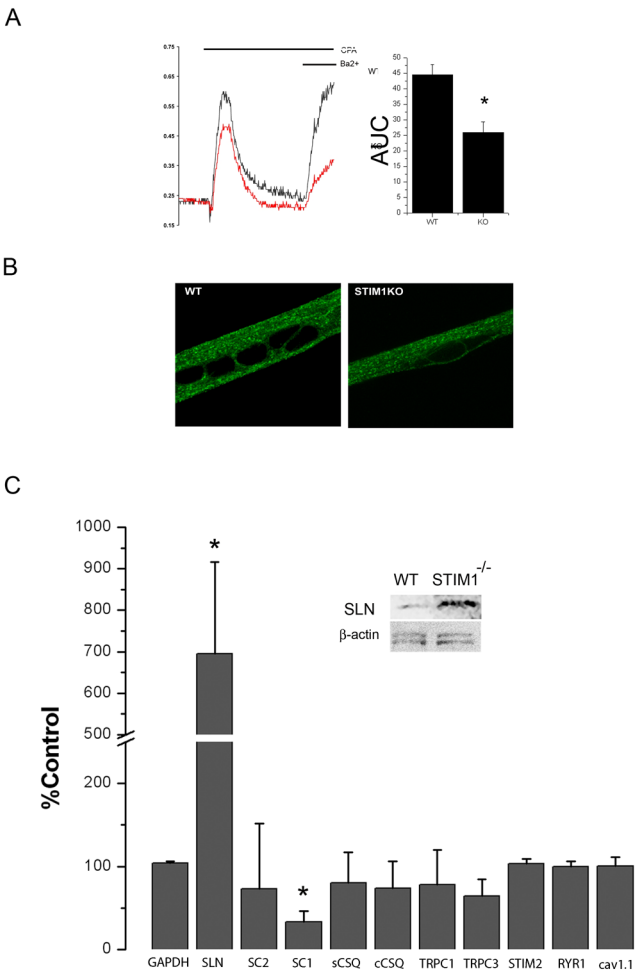

Figure 2.

SR Ca^{2+} stores are reduced in *STIM1* $^{-/-}$ myotubes. A) Myotubes from *STIM1* $^{-/-}$ neonates and WT littermates were perfused in a zero calcium solution and subjected to store depletion with CPA for measurement of internal Ca^{2+} store content. Following store depletion, 2 mM Ba^{2+} was added as a surrogate of calcium for measurement of SOCE. *STIM1* $^{-/-}$ myotubes exhibited decreased internal Ca^{2+} store content and absent SOCE. Myotubes lacking functional STIM1 exhibited markedly reduced SR Ca^{2+} content compared to WT myotubes as measured by AUC of the Ca^{2+} transient following CPA treatment. B) Immunostaining with a CSQ specific antibody revealed no SR morphologic differences between *STIM1* $^{-/-}$ and WT myotubes. C) Expression profiling by RT-PCR of neonatal skeletal muscle from *STIM1* $^{-/-}$ mice and WT littermates (n=4 for both WT and *STIM1* $^{-/-}$). We observed a significant increase in expression of SLN which was confirmed by Western blotting (see inset). There was a decrease in expression of SERCA1 (SC1), with no detectable changes in SERCA2 (SC2), skeletal or cardiac isoforms of calsequestrin (sCSQ, cCSQ). No differences were detected in the mRNA transcripts for STIM2, RYR1, Cav1.1 or TRPC1/3 channels between the WT and $STIM1^{-/-}$ muscle (* $p < 0.05$).

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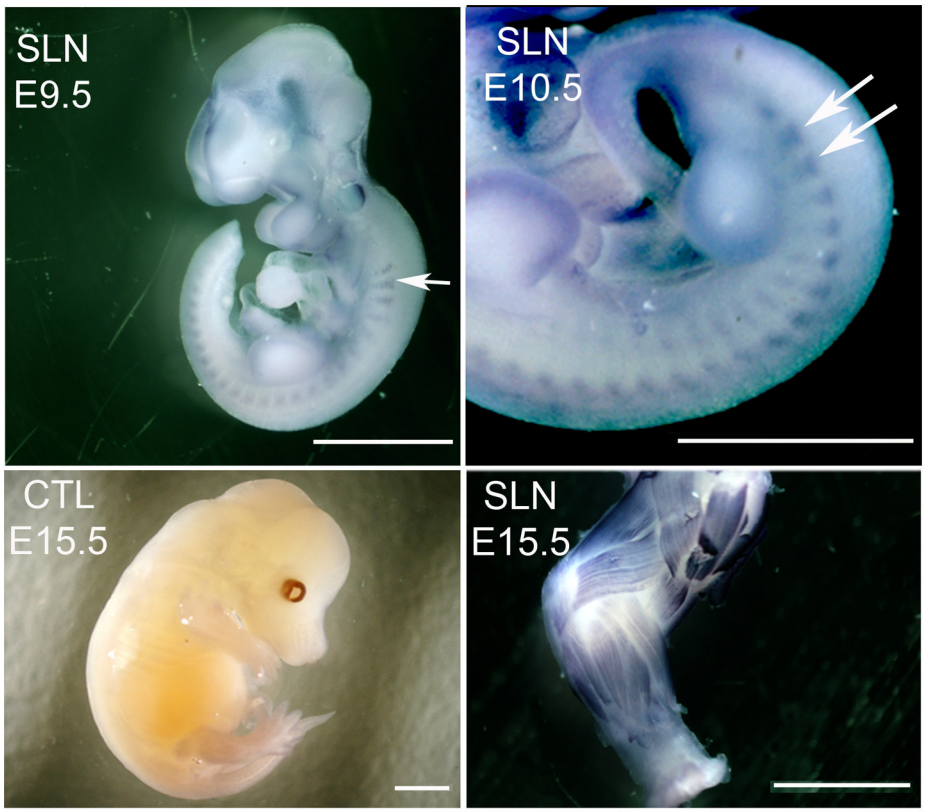

Figure 3.

SLN is expressed in embryonic muscle. *In situ* hybridization using an anti-sense probe showing SLN expression during embryonic development (E9.5 and E10.5, upper panels). Arrows highlight expression in somites. *In situ* hybridization (E15.5) using a SLN sense control probe (lower left panel). Hybridization with an anti-sense SLN probe revealed that SLN mRNA expression is widely expressed in muscle tissues at E15.5 (lower right panel). Isolated hindlimb is shown with skin removed prior to hybridization. (Scale bars = 100 μM top panels, 1 mm bottom panels).

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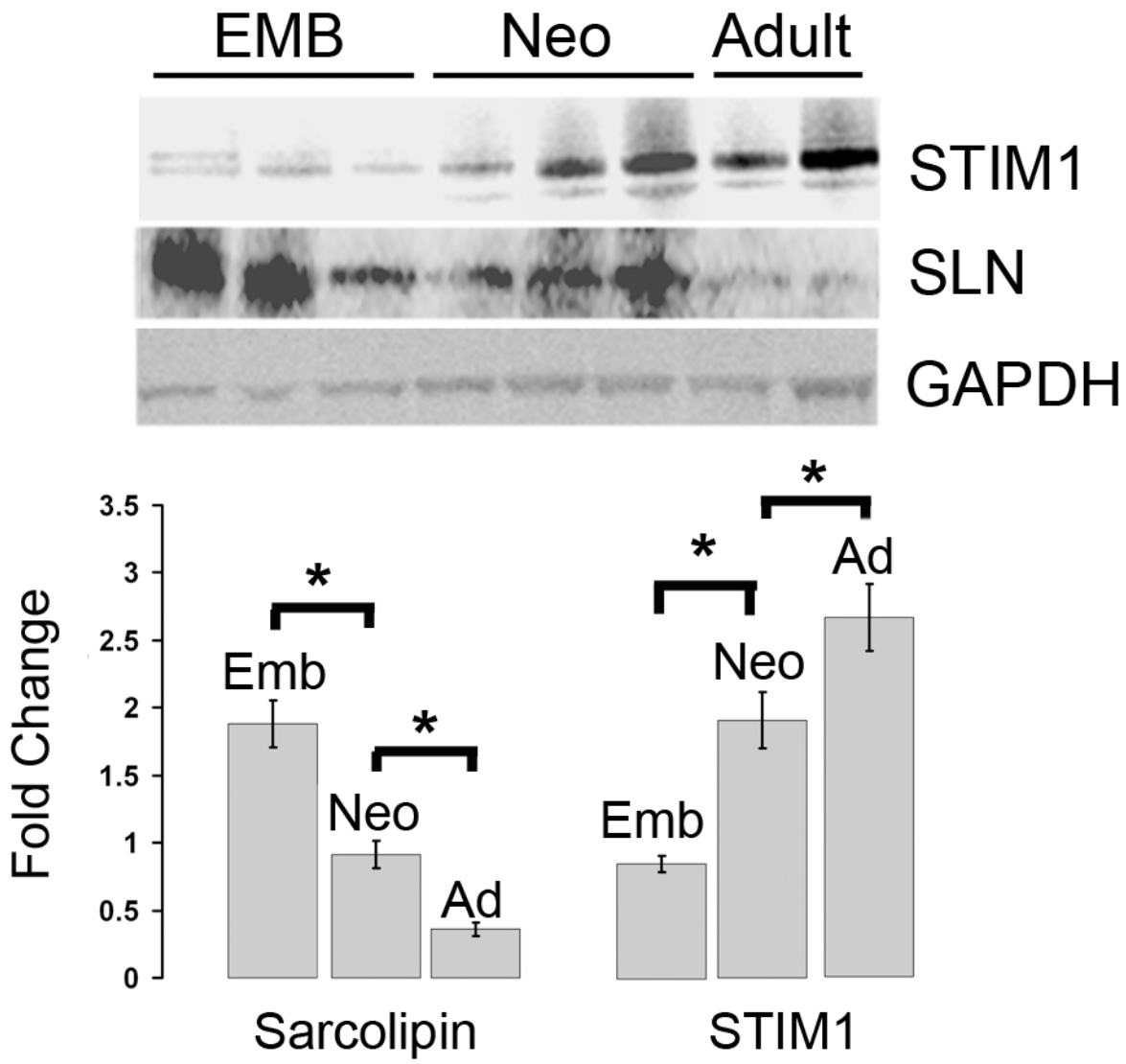

Figure 4.

Western blotting of skeletal muscle lysates showing temporal changes in expression of STIM1 and SLN between embryonic (EMB), neonatal (Neo), and adult (Ad) skeletal muscle. Representative western blot is shown. Bar graph shows quantification of western blotting for the different developmental stages. Hindlimb muscles were harvested from embryos and neonates. The gastrocnemius muscle was isolated from muscles of adult mice. This experiment was repeated three separate times (n=5 for each time point, * $p < 0.05$).

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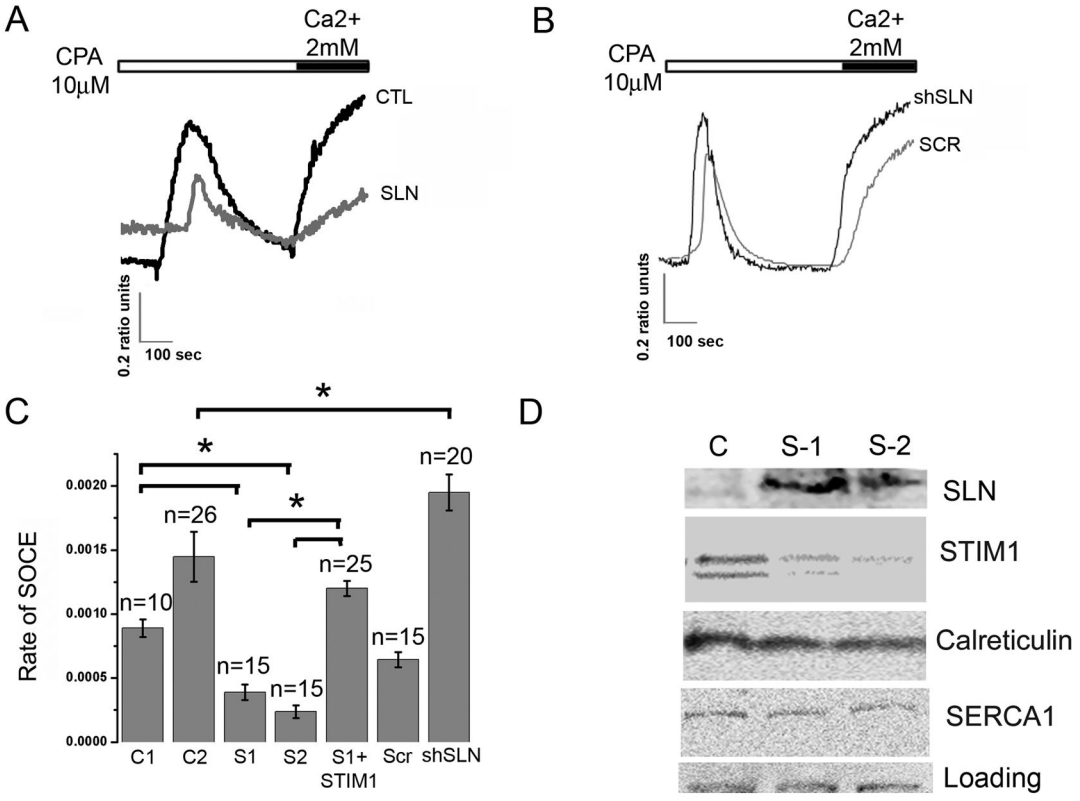

Figure 5.

SLN suppresses SOCE in cultured myotubes. A) Calcium transients showing a reduction in Ca^{2+} store content and decreased SOCE in myotubes stably expressing SLN and IRES-YFP (gray trace) compared to control myotubes expressing empty vector and IRES-YFP (black trace). Representative mean traces are shown. B) Augmented SOCE in myotubes transiently transfected with a shRNA plasmid designed to silence endogenous SLN (black trace) compared to myotubes transfected with a scrambled control plasmid (gray trace). Cells were co-transfected with YFP (1:3, YFP:shRNA) to identify cells for imaging. C) Rate of SOCE as measured by the slope of the transient during Ca^{2+} add back following store depletion: control cell lines (C1 and C2), cell lines stably expressing SLN (S1 and S2), cell lines stably expressing SLN which were then transfected with STIM1 (S1+), cells transfected with scrambled control plasmid (Scr), and SLN silencing plasmid (shSLN) (* $p < 0.05$). D) Western blotting showing levels of expression of SLN and STIM1 in myotubes stably overexpressing SLN (S1 and S2) and controls. No change was noted in levels of expression of calreticulin and SERCA1.

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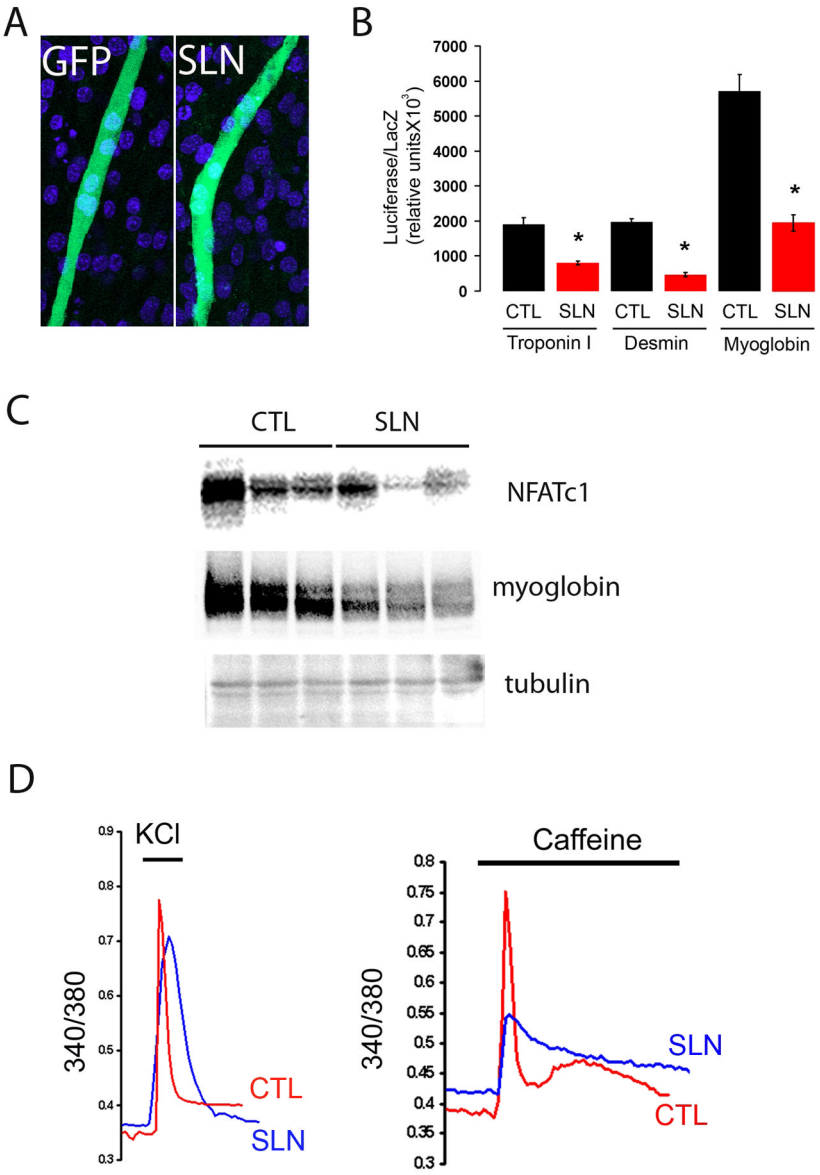

Figure 6.

SLN overexpression suppresses muscle differentiation. A) Confocal images of myotubes transfected with SLN-IRES-GFP and control myotubes transfected with IRES-GFP. There was no significant effect on morphology. B) Myotubes (SLN and control) were transfected with reporter plasmids carrying luciferase downstream of either various muscle specific promoters (desmin, myoglobin and slow troponin I) or a basal TATA construct. Cells were co-transfected with pEF-LacZ to control for transfection efficiency. Cells were differentiated for 4 days in culture into multinucleated fused myotubes. Cell lysates were then assayed for luciferase and ß-galactosidase. Reporter assays showed decreased activation of desmin, slow troponin and myoglobin promoters in the myotubes overexpressing SLN. No change was noted in activation of a basal TATA promoter (not shown). Studies were done in triplicate (*, $p<0.01$). C) Western blotting showing decreased expression of myoglobin and NFATc1 in myotubes stably expressing SLN compared with controls. D) Ca^{2+} transients of SLN overexpressing (blue trace) and control myotubes (red trace) in response to KCl stimulation

(40 mM) and caffeine (20 mM). Representative average traces from five separate experiments are shown.

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Figure 7.

Regulation of calcium signaling by SLN and STIM1 during muscle differentiation. Embryonic muscle expresses high levels of SLN and relatively low levels of STIM1 resulting in decreased SR Ca^{2+} store content and reduced SOCE. In adult muscle, SLN expression is attenuated and STIM1 is upregulated, resulting in a mature SR with increased SR Ca^{2+} store content and fully intact SOCE.